Epigenetic Attenuation of Mitochondrial Superoxide Dismutase 2 in Pulmonary Arterial Hypertension
A Basis for Excessive Cell Proliferation and a New Therapeutic Target

Stephen L. Archer, MD; Glenn Marsboom, PhD; Gene H. Kim, MD; Hannah J. Zhang, PhD; Peter T. Toth, PhD; Eric C. Svensson, MD, PhD; Jason R.B. Dyck, PhD; Mardi Gomberg-Maitland, MD; Bernard Thébaud, MD; Aliya N. Husain, MD; Nicole Cipriani, MD; Jalees Rehman, MD

Background—Excessive proliferation and impaired apoptosis of pulmonary artery (PA) smooth muscle cells (PASMCs) contribute to vascular obstruction in patients and fawn-hooded rats (FHRs) with PA hypertension (PAH). Expression and activity of mitochondrial superoxide dismutase-2 (SOD2), the major generator of H2O2, is known to be reduced in PAH; however, the mechanism and therapeutic relevance of this are unknown.

Methods and Results—SOD2 expression in PASMCs is decreased in PAH patients and FHRs with PAH. FHR PASMCs have higher proliferation and lower apoptosis rates than Sprague-Dawley rat PASMCs. Moreover, FHR PASMCs have hyperpolarized mitochondria, low H2O2 production, and reduced cytoplasmic and mitochondrial redox state. Administration of SOD2 small interfering RNA to normal PASMCs recapitulates the FHR PAH phenotype, hyperpolarizing mitochondria, decreasing H2O2, and inhibiting caspase activity. Conversely, SOD2 overexpression in FHR PASMCs or therapy with the SOD-mimetic metalloporphyrin Mn(III)tetrakis (4-benzoic acid) porphyrin (MnTBAP) reverses the hyperproliferative PAH phenotype. Importantly, SOD-mimetic therapy regresses PAH in vivo. Investigation of the SOD2 gene revealed no mutation, suggesting a possible epigenetic dysregulation. Genomic bisulfite sequencing demonstrates selective hypermethylation of a CpG island in an enhancer region of intron 2 and another in the promoter. Differential methylation occurs selectively in PAs versus aortic SMCs and is reversed by the DNA methyltransferase inhibitor 5-aza-2'-deoxycytidine, restoring both SOD2 expression and the ratio of proliferation to apoptosis. Expression of the enzymes that mediate gene methylation, DNA methyltransferases 1 and 3B, is upregulated in FHR lungs.

Conclusions—Tissue-specific, epigenetic SOD2 deficiency initiates and sustains a heritable form of PAH by impairing redox signaling and creating a proliferative, apoptosis-resistant PASMC. SOD augmentation regresses experimental PAH. The discovery of an epigenetic component to PAH may offer new therapeutic targets. (Circulation. 2010;121:2661-2671.)

Key Words: epigenesis, genetic ■ gene silencing ■ hypoxia-inducible factor-1, α subunit ■ hypertension, pulmonary ■ mitochondrial diseases ■ oxidation-reduction ■ superoxide dismutase 2

Pulmonary artery (PA) hypertension (PAH) is a syndrome in which obstructed, constricted small PAs and increased pulmonary vascular resistance ultimately lead to right ventricular hypertrophy and failure. Despite important advances in understanding the mechanism of PAH, such as the discovery of mutations in bone morphogenetic protein receptors in familial PAH, and the advent of effective oral therapies, such as phosphodiesterase-5 inhibitors and endothelin antagonists, mortality remains high (15% at 1 year).

Clinical Perspective on p 2671

PAH may be viewed in part as a disease of excess proliferation and impaired apoptosis of the PA smooth muscle cell (PASMC), as reviewed elsewhere. Although this study focuses on PASMCs, many cell types are abnormal in PAH. There is infiltration of the lung with inflammatory cells, disorganized endothelial proliferation in plexiform lesions, activation of myofibroblasts, and disruption of the extracellular matrix. The animal model used in this study (the fawn-hooded rat [FHR]) develops spontaneous PAH, characterized by excessive proliferation and impaired apoptosis of the PASMCs; therefore, this model is useful for studying the role of PASMCs in PAH. Moreover, FHRs share with human PAH PASMCs a disrupted mitochondrial network and low expression and activity of superoxide dismutase-2 (SOD2).
The proliferation/apoptosis imbalance in PAH suggests similarities to cancer, although it should be noted that endothelial cells and PASMCs do not invade the surrounding tissue or metastasize. Nevertheless, FHR PAH and certain cancers share a metabolic profile that creates a “pseudo-hypoxic” cellular environment that favors proliferation. Relevant abnormalities include decreased SOD2, normoxic activation of the transcription factor hypoxia-inducible factor-1α (HIF-1α), and HIF-1α-induced activation of pyruvate dehydrogenase kinase, which inhibits the Krebs cycle, shifting metabolism toward glycolysis. As a consequence, electron flux is reduced, and generation of reactive oxygen species (ROS) in the mitochondria decreases.

There is evidence that implicates SOD2 in the development of idiopathic PAH. SOD2 downregulation precedes PAH in FHRs. In rats, the SOD2 gene is located on chromosome 1 and FH-BN1 consomic rats (identical to FHRs except they have introgression of a normal chromosome 1) do not develop PAH. Likewise, in human PAH, SOD2 deficiency is noted in PAs and plexiform lesions. Biological plausibility for a causal role for SOD2 is supported by the recent recognition that SOD2 is a putative tumor-suppressor gene and that epigenetic silencing of SOD2 increases proliferation of cancer cells.

SOD2 is the gatekeeper that regulates physiological production of H2O2 (produced from mitochondrial superoxide during respiration). H2O2 is less toxic than superoxide, and its greater diffusion radius allows it to serve as a signaling molecule. H2O2 modulates the activity of transcription factors such as HIF-1α (which it inhibits) and sulfhydryl rich proteins, including the voltage-gated potassium channel Kv1.5 (which it activates). Because FHR PAH is heritable and yet sequencing showed that the SOD2 gene has no mutations, we hypothesized that epigenetic suppression of SOD2 may initiate and/or sustain PAH. We report here that selective hypermethylation of CpG islands in the SOD2 gene promotes a proliferative, antiapoptotic phenotype in PASMCs and regresses PAH in vivo. This epigenetic mechanism may have significant mechanistic and therapeutic implications in human PAH.

Methods

Animal Studies

The University of Chicago Animal Care Committee approved all protocols. FHRs were purchased from Charles Rivers Laboratories (Wilmington, Mass) and then bred in our facility. FHRs were studied at ~40 weeks of age. Age-matched FH-BN1 rats and weight-matched Sprague-Dawley rats (SDRs) served as controls.

ROS in Isolated Resistance PAs and PASMCs

ROS were measured in isolated resistance PAs with L-012 chemiluminescence or electron paramagnetic resonance.

Electron Paramagnetic Resonance

Isolated resistance PAs were incubated with a derivative of cyclic hydroxylamine (200 μmol/L in Krebs-HEPES buffer with desferrioxamine and DETC at 37°C). Changes in ROS production during a 5-minute acquisition period were quantified by measuring the peak height of electron paramagnetic resonance triplet signal, a measure of 3-methoxy carbonyl-proxyl nitroxide kinetics.

Enhanced Chemiluminescence

The PA was incubated in L-012 (100 μmol/L for 30 minutes at 37°C; Wako Chemicals, Richmond, VA), a superoxide-sensitive chemiluminescence enhancer that does not undergo redox cycling.

Amplex Red

Measurement of H2O2 production was performed in PASMC or PA rings with the fluorometric Amplex Red assay.

Immunoblotting and Immunofluorescence

Immunoblotting was performed on 25 μg protein pooled from n = 3 fourth division PAs or isolated PASMCs (see the online-only Data Supplement).

Small Interfering RNA

PASMCs were grown to ~50% confluence and then exposed to anti-SOD2 small interfering RNA (siRNA). Several “on-target” siRNAs (Applied Biosystems, Foster City, Calif) were tested, and the optimal dose to achieve maximal knockdown with minimal toxicity was established. For each experiment, there was a scrambled siRNA control. Details are given in the online-only Data Supplement.

Replication-Deficient Adenoviruses

FHR PASMCs were infected with serotype 5 recombinant adenovirus carrying the human SOD2 gene under a cytomegalovirus promoter as described (see the online-only Data Supplement).

Redox-Sensitive Green Fluorescence Protein Constructs

Redox-sensitive green fluorescence protein (GFP) constructs (roGFP2) targeted to either the cytoplasm or mitochondria (a kind gift of Dr Remington, University of Oregon) were transfected into PASMCs with the Fugene HD transfection reagent (Roche, Basel, Switzerland). By using different excitation wavelengths (400 and 490 nm) and measuring emission at 535 nm, we assessed the redox status of cells (the greater light emission, the more oxidized the PASMCs).

O2 Consumption

O2 consumption was measured with a Mitocell MT200 chamber and 782 oxygen meter (Strathkelvin Instruments, North Lanarkshire, Scotland). Cells (106 in 500 μL) were studied in culture medium at 37°C during a 10-minute protocol.

Metalloporphyrin Mn(III)tetrakis (4-Benzoic Acid) Porphyrin Therapy In Vivo

Metalloporphyrin Mn(III)tetrakis (4-benzoic acid) porphyrin (MnTBAP) is a synthetic, nontoxic, cell-permeable SOD mimetic. A daily 10-mg/kg dose of MnTBAP was selected on the basis of preliminary data and the literature. Similar doses (10 mg/kg IP every 3 days) are well tolerated and prevent proliferation of premalignant cells in a rat model of Barrett esophagus. Once FHRs had established PAH, as confirmed by a PA acceleration time 25 ms, on Doppler echocardiography, they were randomized to receive MnTBAP versus vehicle for 4 weeks. Assessment of functional capacity (a graded treadmill test) and right ventricular mass and function (echocardiography) was performed before and after therapy. The severity of PAH at study termination was determined by Doppler echocardiography and high-fidelity catheterization (44 weeks of age).

Bisulfite Sequencing

Genomic DNA was isolated from resistance PAs of FH-BN1 consomic rats and FHRs (treated with vehicle or 5-aza-2'-
deoxycytidine [5-AZA]; 1 mg/kg body weight IP injection every 3 days for 2 weeks). Genomic DNA was digested with the restriction enzyme HindIII and then modified with sodium bisulfite with the Zymo Research EZ DNA Methylation Kit (Zymo Research, Orange, Calif). We designed primers specific to bisulfite modified DNA in 6 segments of the SOD2 promoter region 2 kb upstream of the transcription initiation site and 1 segment within intron 2 (Table I in the online-only Data Supplement). Polymerase chain reaction products were isolated and purified by agarose gel electrophoresis with a QIAquick Gel Isolation Kit (Qiagen, Valencia, Calif) and cloned into the TOPO TA expression vector (Invitrogen, Carlsbad, Calif). After transformation of 1-shot, chemically competent Escherichia coli cells, 10 to 15 clones were selected for sequencing. Sequences were then analyzed for methylation at all CpG locations.

Statistics
Sample size for all experiments was 10 per group unless stated otherwise. Values are stated as mean±SEM. Intergroup differences between 2 groups were assessed by an unpaired Student t test; an ANOVA with posthoc analysis using the Fisher protected least significance test was used for comparison among multiple groups. Normality was confirmed with a Kolmogorov-Smirnov or D’Agostino and Pearson omnibus test. For data that were not normally distributed (PA muscularization after control or MnTBAP treatment), we used a Mann-Whitney test. A Fisher exact test was used to compare the number of muscularized vessels between MnTBAP and untreated FHRs. A value of P<0.05 was considered statistically significant.

Results
SOD2 Expression Is Reduced in PASMCs of FHRs and Patients With PAH
Immunostaining revealed a marked reduction in SOD2 expression and fragmentation of the mitochondrial reticulum in FHR PASMCs (Figure 1A). In lung tissue harvested at autopsy, SOD2 expression was reduced within the media and adventitia of small PAs and in plexiform lesions (Figure 1B and 1C) of PAH compared with control patients (without lung disease).

SOD2 Knockdown Recapitulates a “PAH Phenotype” in Normal PASMCs
To assess whether downregulation of mitochondrial SOD2 was sufficient to contribute to the initiation of PAH, we used detailed methods for cell culture, quantitative polymerase chain reaction, catheterization, echocardiography, functional capacity (treadmill), PASMC proliferation, and apoptosis assays have been published previously7 and can be found in the Methods section in the online-only Data Supplement. The methodology for quantitative lung histology is also found in the Methods section in the online-only Data Supplement.

The authors had full access to and take full responsibility for the integrity of the data. All authors have read and agree to the manuscript as written.
SOD2 siRNA to reduce SOD2 levels in normal SDR PASMCs. SOD2 siRNA decreased SOD2 expression (mRNA and protein; Figure 2 and Figure I in the online-only Data Supplement). Small interfering SOD2 also decreased H$_2$O$_2$ production (Figure 2A). SOD2 knockdown reduced the production of superoxide in normal PASMCs, as demonstrated by a reduction in L-012 chemiluminescence. The L-012 signal is specifically inhibited by pegylated SOD but not pegylated catalase, confirming that it is a measure of superoxide (Figure II in the online-only Data Supplement). The mitochondrial membrane potential (ΔΨm) of SOD2 siRNA-treated cells became hyperpolarized (Figure 2B). We investigated the effects of SOD2 siRNA on 2 known abnormalities implicated in creating the excess ratio of proliferation to apoptosis in FHR PAH: HIF-1α activation (translocation to the nucleus) and Kv1.5 downregulation.2 SOD2 siRNA activated HIF-1α and downregulated Kv1.5 channel protein expression (Figure 2C and 2D) in SDR PASMCs. The loss of the Kv1.5 channel had 2 consequences associated with apoptosis and increased cell proliferation,7,15 namely increased cytosolic K$^+$ (Figure 2D) and Ca$^{2+}$ (Figure I in the online-only Data Supplement). This elevation of cytosolic K$^+$ was associated with decreased caspase activity in PASMCs (Figure 2D).7 Therefore, reducing SOD2 expression recapitulated many abnormalities seen in FHR PASMCs.

Hypermethylation of the SOD2 Gene in FHRs and the Therapeutic Effects of the Methyltransferase Inhibitor 5-AZA

Sequencing of the entire SOD2 gene, beginning 600 bp upstream from the transcriptional start site in SDRs and FHRs, demonstrated identical sequences (data not shown). This suggested that decreased SOD2 expression in FHRs might reflect epigenetic silencing of gene transcription, as occurs in cancer.16,17 We validated a bisulfite sequencing assay to measure methylation of key regions of the SOD2 gene and examined the effects of demethylation using the methyltransferase inhibitor 5-AZA. Genomic DNA isolated from PAs of FHRs, consomic FH-BN1 rats, and 5-AZA-treated FHRs was analyzed. Although there are many CpG islands in the SOD2 gene, differential hypermethylation (relative to consomic FH-BN1 DNA) was found only in an enhancer region in intron 2 (Figure 3A and 3B). To test the effects of 5-AZA in vivo, we treated FHRs and then isolated genomic DNA from PAs for SOD2 gene methylation analysis. 5-AZA decreased methylation of an intron 2 sequence (Figure 3A and 3B). We next cultured PASMCs from FHRs and SDRs and treated them with 5-AZA. Again, we identified a differentially methylated region within intron 2 (Figure 3C), which was demethylated in response to 5-AZA. There was tissue heterogeneity in the differentially methylated sites, with methylation being present in the SOD2 gene isolated from FHR PA but not in FHR aortas. An additional site of differential methylation was found in the FHR SOD2 promoter (Figure III in the online-only Data Supplement). 5-AZA increased SOD2 mRNA (Figure 3D) and protein (Figure 4A and 4B) in FHR PASMCs in a dose-dependent manner (maximum, 80% SOD2 induction at 10 μmol/L). 5-AZA also restored Kv1.5 expression (Figure 4C).

SOD2 Hypermethylation Promotes a Proliferative, Apoptosis-Resistant Phenotype

Using flow cytometry, we confirmed that under identical conditions FHR PASMCs had roughly double the rate of proliferation and half the rate of apoptosis as SDR PASMCs (Figure 4D and 4E). Remarkably, incubating FHR PASMCs and lung adenocarcinoma A549 cells with 5-AZA for 3 days significantly decreased proliferation and caused a modest increase in apoptosis (Figure 4D and 4E). O$_2$ consumption increased in FHR PASMCs in response to 5-AZA (Figure 4F). These data suggest that methylation-induced SOD2 downregulation creates a low-O$_2$-consumption metabolic state that favors proliferation and suppresses apoptosis.
FHR PASMCs Produce Less ROS and Are in a Reduced State

Baseline L-012 chemiluminescence was lower in FHRs compared with control PASMCs (Figure 5A), and this was mirrored in a reduced redox state in the cytoplasm and mitochondria, as measured with compartment-specific roGFPs (Figure 5B and Figure IV in the online-only Data Supplement). Consistent with this, SOD2 siRNA reduced L-012 chemiluminescence production in PASMCs (Figure IIA in the online-only Data Supplement) and 5-AZA restored ROS production in FHR PAs, as assessed by both chemiluminescence and electron paramagnetic resonance (Figure 5C). Conversely, 5-AZA had no effect on H$_2$O$_2$ production in normal PAs. Control experiments with pegylated SOD and pegylated catalase demonstrate the specificity of our measurements (Figure IIB in the online-only Data Supplement). Thus, there is a reversible, methylation-induced depression of H$_2$O$_2$ production in FHRs.

Increased Expression of DNA Methyltransferases in FHRs

Because gene methylation is dependent on the activity of DNA methyltransferases (MTs), we measured their expression in FHRs and control rats. In lung tissue, both the maintenance DNA MT1 and the de novo DNA MT3B were significantly upregulated compared with control (Figure 6A); a similar pattern was found in PASMCs from FHRs treated with 5-AZA (FHR-Tx; n = 3). Methylation of SOD2 in FHR PASMC is reversible by 5-AZA. A, Schematic of the CG dinucleotide percentage and CpG islands within the SOD2 promoter and first 2 kb after the transcriptional start site. Seven amplicons were surveyed within the SOD2 gene. Their approximate locations are represented by solid horizontal lines. The positions of individual CpG dinucleotides are shown as vertical tick marks below the amplicon map. No methylated CpG pairs were identified in amplicons 3 to 6. Differentially methylated CpG dinucleotides in FHRs within amplicon 7 vs BN1 tissue. B, Corresponding methylation percentage of the differentially methylated CpG in intron 2. Results are expressed as a frequency of cytosine methylation in PAs from consomic control BN1 rats (n = 2), FHRs (n = 3), and FHRs treated with 5-AZA (FHR-Tx; n = 3). C, Representative sequencing traces of genomic DNA from cultured PASMCs. Only methylated cytidines are protected against bisulfite-mediated deamination of cytidine into uridine (which is recognized as thymidine when the polymerase chain reaction product is amplified). As indicated by the arrow, the cytidine in FHR PASMCs was methylated (and therefore remains a cytidine; top left); this is reversed by 5-AZA. The site is not methylated in FHR aortic SMCs or in SDR PASMCs. The bar graph shows the mean data indicating the reversibility and tissue specificity of this SOD2 methylation in intron 2 in cultured PASMCs. D, FHR PASMCs have lower SOD2 mRNA levels vs consomic PASMCs. 5-AZA causes a dose-dependent increase in SOD2 expression.

SOD2 Augmentation Restores FHR PASMC Gene Expression

Because 5-AZA is a nonspecific demethylating agent and may affect a multitude of genes, we investigated the thera-
peutic effects of direct SOD2 augmentation to assess its role in PAH. We used 2 complementary strategies: direct SOD2 augmentation with adenoviral gene transfer carrying mitochondrialy targeted SOD2 and administration of a membrane-permeable SOD analog, MnTBAP.

Figure 4. 5-AZA restores SOD2 expression, reduces proliferation, and enhances apoptosis in FHR PASMCs. A and B, Representative and mean data showing that FHR PASMCs express less SOD2 than SDR PASMCs; this is partially reversible with 5-AZA treatment. n=4 per group. *P<0.05. C, 5-AZA-induced, dose-dependent increase in Kv1.5 mRNA in FHR PASMCs. *P<0.05. D and E, 5-AZA (black bars) increases apoptosis and decreases proliferation in FHR PASMCs and lung cancer cells (A549) but not SDR PASMCs. n=4 to 6 per group. *P<0.05 between baseline and 5-AZA. †Significant difference from SDR PASMCs. F, 5-AZA increases O2 consumption in FHR and SDR PASMCs but not A549 cells (n=3 to 8 per group).

Adenoviral SOD2 significantly increased SOD2 expression in FHR PASMCs (Figure 7A). This inactivated the spontaneously active HIF-1α seen in FHRs and increased Kv1.5 expression (Figure 7B). Likewise, incubation of FHR PASMCs with MnTBAP for 48 hours inactivated nuclear

Figure 5. Lower ROS levels and reduced cellular environment in FHR PASMCs. A, Decreased ROS levels in freshly isolated resistance PAs from FHRs vs consomic rats measured with L-012 chemiluminescence at 37°C. B, PASMCs were transfected with redox-sensitive GFP constructs targeted to the cytoplasm (top) and mitochondria (bottom). FHR PASMCs had reduced cytoplasm and mitochondria relative to SDR PASMCs. Dithiothreitol and tert-butyl hydroperoxide were used to completely reduce and oxidize the cells, respectively. C, ROS mean data and representative trace (inset) showing the cyclic hydroxylamine (CMH) triplet signal. Peak height (electron paramagnetic resonance [EPR] amplitude units) is proportional to ROS levels and is normalized to wet weight. PAs from FHRs treated with 5-AZA (black bars) have increased production of ROS (left) and H2O2 (right) vs untreated FHRs. Control rats show no induction of ROS or H2O2, n=5 per group.
HIF-1α (Figure 7C and 7D) and restored Kv1.5 expression (Figure 7D).

SOD2 Augmentation Regresses PAH In Vivo
To test the therapeutic effects of SOD2 augmentation in vivo, we treated FHRs with proven PAH with MnTBAP. MnTBAP was chosen over SOD2 because of the very transient upregulation of transgene expression (~2 weeks) that is often found with adenoviral gene therapy. MnTBAP doubled PA acceleration time (Figure 8A) and caused a concomitant reduction in the mean PA pressure (Figure 8B) and right ventricular free wall thickness (Figure 8A). During the 4-week treatment, exercise capacity deteriorated in vehicle-treated FHRs, whereas the MnTBAP group showed improvement (Figure 8C). Quantitative histology performed by blinded readers demonstrated that MnTBAP caused a significant reduction in the percent medial thickness of precapillary resistance arteries and an increase in the percentage of small (25 to 50 μm) nonmuscular PAs (Figure 8D).

Discussion
One of the major findings of this study is the identification of SOD2 methylation as a potential new, epigenetic mechanism for PAH in an animal model of spontaneous and heritable PAH. This may be relevant for clinical PAH because SOD2 is also downregulated in human PAH (Figure 1). We identified hypermethylation of a CpG island in an enhancer region within intron 2 and the promoter of SOD2 as the basis for SOD2 downregulation in FHRs. This appears to reflect significantly higher expression of DNA MT1 and MT3B in lungs and of DNA MT3B in isolated PASMCs of FHRs (Figure 6). This epigenetic downregulation of SOD2 impairs H₂O₂-mediated redox signaling, activates HIF-1α, and creates a proliferative, apoptosis-resistant state (Figures 4, 5, and 7). Both the mechanism of SOD2 downregulation and its consequences parallel those recently discovered in human cancer, but to the best of our knowledge, this is the first report of an epigenetic cause of a pulmonary vascular disease. The epigenetic downregulation of SOD2 was reversible by treatment with the DNA MT inhibitor 5-AZA. Furthermore, our study demonstrates that augmentation of SOD2 (by 3 complementary strategies) restores mitochondrial function, inhibits PASMC proliferation, and increases cell apoptosis in vitro. In vivo, MnTBAP causes partial regression of established PAH and decreases the muscularization of pulmonary precapillary resistance vessels (Figure 8A and 8D).

The FHR SOD2 deficiency is the consequence of the covalent cytosine methylation that occurs in dinucleotide CpGs (Figure 3). Methylation at the C5 atom of cytosine inhibits gene expression by preventing the binding of transcription factors. Methylation is reversible and heritable and can be tissue specific. Indeed, SOD2 methylation occurred in the FHR PA but not in the FHR aorta (Figure 3), thus pointing to tissue-specific epigenetic mechanisms. This finding may contribute to our understanding of the localization of the pathology in human and FHR PAH to the pulmonary circulation. The SOD2 promoter and several of its introns have CpG islands that offer potential methylation sites. Using genomic bisulfite sequencing, we directly confirmed that FHRs have 2 discrete sites of differential hypermethylation (1 in intron 2, 1 in the promoter; Figure 3A through 3C and

Figure 7. SOD2 supplementation inactivates HIF-1α and increases Kv1.5 expression in FHR PASMCs. A, The SOD2 virus and a control GFP virus are effective, increasing expression of SOD2 and GFP mRNA, respectively, in FHR PASMCs. B, Immunofluorescence with an Alexa647 secondary antibody shows that the SOD2 adenovirus (Ad-) increases human SOD2 expression (green; bottom left). This restores Kv1.5 expression (green) and inactivates HIF-1α (red nuclei). Nuclei are counterstained blue (DAPI). C and D, A 48-hour incubation with MnTBAP decreases nuclear localization of HIF-1α and restores Kv1.5 expression.
Figure III in the online-only Data Supplement). Although many regions in the SOD2 gene are heavily methylated, these 2 were the only CpG islands that were both differentially methylated and demethylated by 5-AZA. The intron 2 site is in the same region of the SOD2 gene that has been identified in transformed human lung fibroblast cell lines.20 5-AZA covalently binds and irreversibly inhibits DNA MTs,21 resulting in reactivation of transcription of previously methylated genes after a requisite cell division. It is unclear why 5-AZA had no effect on other areas of methylation, but other studies have described similar observations.17

CpG methylation is established and maintained by 3 DNA MTs.22 DNA MT1 is expressed in proliferating tissues, and its activity is coupled to DNA replication, copying methylation patterns from the parental to the daughter strand. Therefore, it is considered primarily a maintenance MT. In contrast, DNA MT3A and MT3B are considered de novo MTs that can establish new methylation patterns. We identified an upregulation of DNA MT3B in FHR PASMCs (Figure 6), which would be an elegant explanation for the observed hypermethylation of the SOD2 gene. Interestingly, DNA MT3B depletion is sufficient to reactivate methylation-silenced genes and to decrease proliferation in both human breast adenocarcinoma and A549 cells.23

It is interesting that changes in the activity or expression of DNA MTs can have a relatively specific outcome in terms of site-specific methylation and regulation of specific genes in certain tissues. Current models suggest that the specificity of DNA MT activity can depend on their expression levels or their interaction with other epigenetic regulators. In the present study, for example, we found that FHR animals with PAH had higher expression levels of DNA MT3B. This DNA MT has been shown to have distinct CpG methylation activity patterns that depend on the tissue environment and on the expression of differentially spliced isoforms.24 Another level of DNA methylation specificity is achieved by the interaction of DNA MTs with other epigenetic regulators such as histone deacetylases. For example, DNA MT3B contains an ATRX homology domain that interacts with histone deacetylase 1.25 Interestingly, such an association between increased CpG methylation and decreased histone acetylation has been observed for the SOD2 gene.16

Gene methylation has an established role in promoting pathological cell proliferation in cancer. SOD2, a candidate tumor-suppressor gene,8,9 is silenced in several malignancies,16,17,26 In multiple myeloma and pancreatic carcinoma, the epigenetic silencing of SOD2 is caused by hypermethyl-
Demethylation of SOD2 in cancer restores SOD2, increases H$_2$O$_2$, and decreases cell proliferation and tumor growth, consistent with our observations (Figures 3D, 4D, and 4E). The effects of directly overexpressing SOD2 (by 3 complementary means) are concordant with the effects of SOD2 demethylation in cancer (Figure 7A and 7B). Our study demonstrates that reversing gene methylation is beneficial in FHR PASMCs in vitro. Treatment with 5-AZA decreases SOD2 methylation and causes a dose-dependent increase in SOD2 and Kv1.5 expression in FHR PASMCs (Figure 4A through 4C).

Production of H$_2$O$_2$ is a critical link between SOD2 expression and regulation of proliferation (Figure V in the online-only Data Supplement provides a schematic representation of the cascade of the proposed transcriptional, metabolic, and redox consequences of SOD2 downregulation). Simply knocking down SOD2 expression in a normal PASMC diminishes endogenous H$_2$O$_2$ production and leads to an associated activation of HIF-1α (Figure 2). Moreover, an anti-SOD2 siRNA caused many of the other abnormalities seen in human and FHR PAH, including a decrease in expression of Kv1.5 channels and a rise in cytosolic calcium (Figure IB in the online-only Data Supplement). Conversely, augmenting SOD2 elevates production of H$_2$O$_2$ in FHR PAs (Figure 5C) and reduces cell proliferation (Figure 4E). Similarly, in prostate cancer, overexpressing SOD2 increases H$_2$O$_2$ and reduces cell proliferation. Thus, in both PAH and cancer, there is an inverse correlation between SOD2 activity and H$_2$O$_2$-mediated cell proliferation. Catalase, which reduces H$_2$O$_2$ levels, prevents the ability of SOD2 to inhibit cancer cell proliferation. These observations suggest that the effect of SOD2 augmentation in PAH and cancer is mediated by increasing H$_2$O$_2$ production (from a deficient starting level). We support this contention by showing that 5-AZA increases H$_2$O$_2$ production only in FHR PASMCs, having little effect on normal PASMCs (Figure 5C).

Our study also identifies the downstream mechanism by which this mitochondrial abnormality promotes cell proliferation, namely normoxic activation of HIF-1α. HIF-1α activation has previously been identified in human PAH and FHR PAH. The present study supports a key role for H$_2$O$_2$ as the link between SOD2 and cell proliferation, as schematized in Figure V in the online-only Data Supplement. Although ROS are toxic at high levels, there is a physiological level of H$_2$O$_2$ production by mitochondria during normal oxidative metabolism. Physiological levels of H$_2$O$_2$ serve as redox signaling molecules, involved in oxygen sensing. The evidence linking SOD2 levels to ROS in PAH is clear. Simply lowering SOD2 message levels (with siRNA) decreases mitochondrial H$_2$O$_2$ production (Figure 2A); conversely, demethylating the SOD2 gene enhancer in FHR PAs restores SOD2 expression, leading to increased ROS and mitochondrial H$_2$O$_2$ production (Figure 5C). Compartment-specific, redox-sensitive GFPs demonstrated that the net change in redox state in FHR (versus SDR) PASMCs is reduction (in both the cytosol and mitochondria), consistent with the observed decreased ROS levels in FHRs (Figure 5).

**Therapeutic Implications**

Evidence for the relevance of SOD2 deficiency in PAH comes from the concordant benefit of 3 different strategies that restore SOD2 expression or SOD activity in our study. In FHR PASMCs, SOD2 gene therapy, application of the SOD2 mimetic MnTBAP, or 5-AZA each led to HIF-1α inactivation and restoration of Kv1.5 expression (Figure 7). Although these surrogate end points are important, the key therapeutic finding of our study is that MnTBAP treatment regresses PAH in vivo (Figure 8A and 8D). This hemodynamic benefit is associated with a reduction in right ventricular hypertrophy and improved functional capacity and lung histology. The concordant findings in response to complementary strategies to modulate SOD2 expression reduce the possibility of artifacts related to flaws in any single strategy such as the stress of intraperitoneal MnTBAP injections, inflammation from the SOD2 adenovirus, and potential confounding effects of demethylation of nontarget genes by 5-AZA.

The benefits of MnTBAP are consistent with those of tempol (another membrane permeable SOD mimetic), which decreases hypoxic pulmonary hypertension in rats, and recombinant SOD1, which ameliorates persistent pulmonary hypertension in newborn lambs. Bowers et al. have also noted decreased SOD2 in the PAs in human PAH; however, their interpretation was that PAH is a condition of increased oxidative stress (in part because other oxidant markers were elevated). Our data indicate that in FHRs, H$_2$O$_2$ is subphysiological because of both a primary effect, methylation-induced reduction of the SOD2 gene expression, and a secondary effect, pyruvate dehydrogenase kinase–mediated inhibition of oxidative metabolism. Identification of the therapeutic potential for 5-AZA is particularly relevant because 5-AZA (decitabine) is approved for human use in myeloproliferative disorders (where it demethylates p15).

**Limitations**

There are limitations in using human tissue. One notable limitation is that we are unsure about smoking status. We acknowledge that smoking could change SOD2 levels; however, this is unlikely to be an important confounder because the rodent data are consistent with the human data. We acknowledge that the net effect of loss of SOD2 is controversial (oxidation in SOD2 knockout mice versus reduction in our study). Interestingly, the SOD2 haploinsufficient mouse has a doubling of cancer risk, supporting our central finding that downregulation of this mitochondrial enzyme is a proliferative, antiapoptotic signal. We suspect the differences in ROS relate to the lifelong duration and more profound severity of SOD2 loss for the knockout mouse compared with acquired and more modest SOD2 loss in FHRs.

Finally, pyruvate dehydrogenase kinase activation in PAH thwarts mitochondrial respiration, limiting input to the mitochondrial electron transport chain. Although in FHRs low ROS and a reduced state activate HIF-1α, others find that high ROS levels stabilize HIF-1α. Interestingly, Kaewpila et al. noted in cancer that siSOD2 activates HIF-1α, consistent with our findings. However, they found that siSOD2 increased super-
oxide levels without changing H$_2$O$_2$ levels. This again may relate to the magnitude of the SOD2 decrease achieved, the cell-specific differences in metabolic activity and prolyl hydroxylase activity, and the status of the many other antioxidant systems. Our findings are supported by earlier studies showing that short preexposure of cells to H$_2$O$_2$ selectively prevents hypoxia-induced accumulation of HIF-1α protein.\textsuperscript{10}

Finally, PHR are unusually sensitive to environmental hypoxia. In this regard, it is noteworthy that much of the study was conducted in Edmonton, Alberta (elevation of 2372 feet above sea level). Altitude may be an important factor in determining the time to onset of PAH and severity of the disease in FHR.

**Conclusion**

The recognition of a novel epigenetic mechanism of PAH (methylation-induced attenuation of SOD2 expression) may partially explain the excessive cell proliferation and decreased apoptosis in PAH and could offer new therapeutic targets.

**Sources of Funding**

This work is supported by National Institutes of Health grants RO1-HL071115 and 1RC1HL099462-01, the American Heart Association, and the Roche Foundation for Anemia Research.

**Disclosures**

None.

**References**


**CLINICAL PERSPECTIVE**

Pulmonary arterial hypertension (PAH) is characterized by remodeling of small precapillary resistance arteries, leading to increased vascular resistance and right ventricular failure. PAH is increasingly seen as a disease in which vascular obstruction is caused not only by vasoconstriction and inflammation but also by enhanced proliferation and impaired apoptosis of vascular cells. The discovery that most patients with familial PAH have mutations in the bone morphogenetic protein receptor type II gene highlighted a potential genetic basis for PAH. However, most sporadic PAH patients do not have these mutations. Here, we report that both fawn-hooded rats (a strain with spontaneous PAH) and humans with PAH have a fragmented mitochondrial network and decreased expression of mitochondrial superoxide dismutase 2 (SOD2) in their pulmonary artery smooth muscle cells. SOD2 is an important generator of hydrogen peroxide, which at physiological levels is a signaling molecule. In cancer, SOD2 is considered a potential oncopogene, and its expression is depressed. Suppression of SOD2 in normal pulmonary artery smooth muscle cells recapitulates the proliferative PAH phenotype. Interestingly, SOD2 downregulation is not due to gene mutation; rather, the SOD2 gene is epigenetically silenced by specific (and reversible) methylation of specific CpG islands in the promoter and intron 2. This epigenetic inhibition of gene transcription is associated with increased DNA methyltransferase expression. SOD supplementation or demethylation strategies reverse the hyperproliferative phenotype of the fawn-hooded rat pulmonary artery smooth muscle cells and regress experimental PAH in vivo. This is the first demonstration of an epigenetic basis for a heritable vascular disease and has pathogenetic and therapeutic implications.
Epigenetic Attenuation of Mitochondrial Superoxide Dismutase 2 in Pulmonary Arterial Hypertension: A Basis for Excessive Cell Proliferation and a New Therapeutic Target

Stephen L. Archer, Glenn Marsboom, Gene H. Kim, Hannah J. Zhang, Peter T. Toth, Eric C. Svensson, Jason R.B. Dyck, Mardi Gomberg-Maitland, Bernard Thébaud, Aliya N. Husain, Nicole Cipriani and Jalees Rehman

_Circulation_. 2010;121:2661-2671; originally published online June 7, 2010; doi: 10.1161/CIRCULATIONAHA.109.916098

_Circulation_ is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2010 American Heart Association, Inc. All rights reserved.
Print ISSN: 0009-7322. Online ISSN: 1524-4539

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://circ.ahajournals.org/content/121/24/2661

Data Supplement (unedited) at:
http://circ.ahajournals.org/content/suppl/2010/06/04/CIRCULATIONAHA.109.916098.DC1

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in _Circulation_ can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to _Circulation_ is online at:
http://circ.ahajournals.org//subscriptions/
Supplemental Methods

**qRT-PCR.** Total RNA is extracted using RNeasy MiniKit (Qiagen). qRT-PCR is performed on 50ng of RNA in 50μl (including primers (500nM) and a TaqMan probe (200nM)) using an ABI PRISM 7700 (Applied Biosystems, Foster City, CA). Reverse transcription proceeds for 30 min at 48°C. AmpliTaq Gold activation occurs for 10 min at 95°C. Forty cycles of PCR are performed. A cycle consists of denaturing (15s at 95°C) and 1 minute of annealing and extension (60°C). $2^{\Delta\Delta Ct}$ is a ratio of the relative expression of the mRNA of interest, normalized to a reporter (e.g. β2microglobulin) and then to a calibrator sample. The primers used have been reported previously¹.

**Hemodynamics.** PAP (pulmonary artery pressure) was measured in closed-chest rats with a 1.4F, micromanometer-tipped catheter (Millar Instruments, Houston, Texas) delivered by a customized introducer-sheath system, without radiologic guidance, as described. Cardiac output was measured by thermodilution and pulmonary vascular resistance is calculated as $\approx c(T_{\text{blood}} - T_{\text{injectate}})V_{\text{injectate}} / \int \Delta T dt$; where $c=1.08$ for D5W & blood; $V_{\text{injectate}} =0.5ml$, and integration period=10s.
**Measurement of functional capacity.** Rats exercised on a calibrated, motorized treadmill (Treadmill Simplex II, Columbus instruments). Incremental increases in treadmill belt speed were made as follows: 1 minute each at 50 and 80m/minute followed by 4 minutes each at 120 and 140m/minute, until exhaustion. Exhaustion was defined as the rats resting >5 consecutive seconds on the shock bar. Prior to data collection, rats were acclimated to the treadmill (in two 3-minute sessions).

**Cardiac ultrasound and anesthesia.** Echocardiography was performed using a Vevo 770 ultrasound system with a 37.5 MHz transducer. Rats were lightly anesthetized with isoflurane (1.6 – 2.0%). Images were recorded at baseline and after 1 month of treatment. Right ventricular thickness and pulmonary artery acceleration time (PAAT), a parameter which varies inversely with mean PAP, were recorded.

**Immunoblotting and immunofluorescence.** Immunoblotting is performed on 25µg of protein pooled from n=3 4th division pulmonary arteries. For immunofluorescence, the primary antibodies used were anti-SOD2 (Upstate, CA) and anti-α-Smooth Muscle Actin (MP Biomedicals, OH). Fluorescent Alexa Fluor-488 and Alexa-568 (Invitrogen, CA) labeled secondary antibodies were imaged on a Zeiss LSM 510 META confocal microscope at 488/490/505LP and 561/565/575-630 (excitation/dichroic/emissions). The mounting medium (Prolong Gold, Invitrogen, CA) contained DAPI which was imaged with a 2P laser.
The thresholded smooth muscle image was binarized and converted to a mask which was used to extract pixel intensity values from the corresponding SOD2 images. After background subtraction, the SOD2 pixel intensities were averaged and these averages (6-6 for large vessels and 9-9 for small vessels) were used to probe for statistical significance.

Muscularization of pulmonary blood vessels. Lungs were perfused with saline and inflated with Z-fix (Anatech, MI) at a pressure of 25cm H$_2$O. After overnight fixation, lungs were paraffin-embedded. Antibodies against von Willebrand factor (Abcam) and anti-$\alpha$-Smooth Muscle Actin (MP Biomedicals, OH) were used. For each animal, at least 40 blood vessels measuring between 25 and 50µm diameter (precapillary resistance vessels) were randomly selected by their von Willebrand factor expression, without knowledge of their muscularization (as shown in Figure 8D, only the red fluorescence channel was used to select a blood vessel and exposure time for the green channel was kept identical for all imaged blood vessels), as described previously$^2$. Images were obtained with a Zeiss Axio Observer Z1 inverted microscope using a LD Plan-Neofluar 40x objective. Morphometric analysis was performed with Zeiss Axiovision 4.6 software. Using the staining for $\alpha$-Smooth Muscle Actin, the outer and inner diameters of the muscular coat were measured and the percentage thickness was calculated as $100\times$(outer diameter-inner diameter)/outer diameter. When blood vessels had an ovoid shape, percentage thickness was determined for both the long and the short axis and then averaged.
**Small interfering RNA (siRNA).** PASMC cells were grown to ~50% confluence and then exposed to anti-SOD2 siRNA. In each case 2-3 “on-target” siRNAs (designed by Applied Biosystems) were tested and dose-response experiments were performed to achieve optimum knockdown of the candidate gene with minimal toxicity. All experiments used ~10 plates of cells/experimental group. For each “on-target” siRNA there was a scrambled siRNA control to assess the specificity of the siRNA effect. FAM fluorophore-labeled siRNAs were designed by Applied Biosystems and transfection rates were ~70%. siRNA is aliquoted into 50µl portions in OptiMEM1 (Applied Biosystems siRNA Transfection II Kit #1631). The culture medium was aspirated from the cell monolayer and the transfection agent (siPORTNeoFX, Ambion siRNA Transfection II Kit #1631) and 60nM siRNA were added, followed by 900µl of OptiMEM1. Cells were incubated at 37ºC for 48h and to ensure gene knockdown, mRNA and protein expression were assessed using qRT-PCR and immunoblotting, respectively\(^3\).

**Caspase activity assays.** PASMCs were either treated with scrambled siRNA or with siRNA against SOD2. Three days later, proteins were isolated and used in a caspase 3 and a caspase 9 colorimetric activity assay (R&D Systems) according to the manufacturer’s instructions. Absorbance at 405nm is normalized to the protein content of the sample and the experiments were repeated 3 times.
**Replication deficient adenoviruses.** A serotype 5 recombinant adenovirus carrying the human SOD2 gene expressed under the control of a CMV promoter was used to infect Fawn-hooded rat PASMCs (passage 1-5) for 6 hours in serum-free medium (multiplicity of infection 100), followed by growth for 48-hours in complete media to allow transgene expression.

**Proliferation and apoptosis assays.** Proliferation (5'-bromo-2'-deoxyuridine, BrdU) and apoptosis (Annexin V), were quantified using flow cytometry. BrdU (1x10^{-5} M), a thymidine analog that incorporates into dividing cells during S phase was added to the media of cells for 1 hour. BrdU (+) cells (fluorescently-labeled with anti-BrdU; BD Pharmingen) were counted. Apoptotic cells bind Annexin V but at the same time maintain membrane integrity. Therefore, apoptotic cells can be distinguished from necrotic cells by their impermeability to the DNA stain propidium iodide.
### Supplemental Table 1. Primer sequences for bisulfite sequencing of the SOD2 gene

<table>
<thead>
<tr>
<th>Segment</th>
<th>Forward primer</th>
<th>Reverse primer</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>AGTTGGGTTGGATATTAGGAT</td>
<td>TCAAAACCTTCTTCAATT TTC</td>
</tr>
<tr>
<td>2</td>
<td>AATATGGTTTGGGGATGAAGTTTT</td>
<td>TCCCCAATTTCTA AAAACTCA</td>
</tr>
<tr>
<td>3</td>
<td>GGGGTTTTTTAGAATTAGGAAT</td>
<td>ACAACTAAACRCTAACCCTAC</td>
</tr>
<tr>
<td>4</td>
<td>TYGTTTTGTATAAGTTGGGT</td>
<td>CTTCTACCCRCCRCTAAAAAA</td>
</tr>
<tr>
<td>5</td>
<td>AAGTGGTAGGTTYGGGG</td>
<td>ACACRAAAACCTCCAATACA</td>
</tr>
<tr>
<td>6</td>
<td>TATTAAYGYGTAGATTATGTTTG</td>
<td>AACTCAACAATAAAAAATACATCAA</td>
</tr>
<tr>
<td>7</td>
<td>TTTATTTTTTATAGGTTTGGGA</td>
<td>AATACCAACCTTTAATTCTCCA</td>
</tr>
</tbody>
</table>
Supplemental Figure 1

**A**  
Superoxide production after SOD2 downregulation  

![Graph A](image)

**B**  
Superoxide production after antioxidant treatment  

![Graph B](image)
Supplemental Figure 2

A

B

[Ca^{2+}]_i (nM)

Control  Scramble  siSOD2

*
Supplemental Figure 3

Cytoplasmic roGFP2

Mitochondrial roGFP2
**Supplemental Figure 1.** A) siRNA mediated downregulation of SOD2 decreases L012 chemiluminescence, indicating a decreased superoxide production in PASMCs (n=4/group). B) Superoxide production after treatment with pegylated SOD and pegylated catalase. Only pegylated SOD significantly reduced superoxide production (n=3/group). * p<0.05.

**Supplemental Figure 2.** A) PASMCs treated with siSOD2 show a strong reduction in SOD2 protein expression. Cells were either treated with scrambled siRNA (upper panels) or siSOD2 (lower panels) and stainings were done 2 days later. SOD2 is shown in green, nuclear DAPI staining is shown in blue. Scale bar = 50 µm. B) Downregulation of SOD2 increases intracellular calcium concentrations.

**Supplemental Figure 3.** Ratios of redox-sensitive GFP emission at 535nm after excitation at 400nm and 490nm. PASMCs were transfected with redox-sensitive GFP constructs targeted to the cytoplasm (left panel) and mitochondria (right panel). Dithiothreitol (DTT) and H₂O₂ were used to completely reduce and oxidize the cells, respectively. FHR PASMC had a reduced cytoplasm and mitochondria, relative to Sprague Dawley PASMC.
Supplemental Figure 4. Schematic representation of the consequences of SOD2 downregulation. Normally, SOD2 rapidly converts superoxide (produced at complexes I and III) to H$_2$O$_2$. Diffusible H$_2$O$_2$ serves as a redox messenger signaling “normoxia”. H$_2$O$_2$ maintains Kv1.5 in an open state and prevents HIF-1$\alpha$ activation. In PAH, SOD2 methylation decreases gene transcription, lowering SOD2 protein and decreasing H$_2$O$_2$ production. This reduces the redox environment, causing HIF-1$\alpha$ activation. HIF-1$\alpha$ induces transcription of pyruvate dehydrogenase kinase (PDK), which inhibits pyruvate dehydrogenase. Inhibition of pyruvate dehydrogenase blocks production of Acetyl-CoA, slowing the Krebs’ cycle and decreasing production of the electron donors. The resulting decrease in passage of electrons down the transport chain further reduces ROS production. This pathologic positive feedback loop can be interrupted by restoring SOD2 activity (using 5-aza-2’-deoxycytidine to demethylate SOD2, SOD2 gene transfer or MnTBAP) or by inhibiting PDK (using dichloroacetate).
